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Molecular Pathogenesis of Liver Disease During Persistent Hepatitis B Virus Infection

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PERSPECTIVE

Introduction of modern methods of molecular biology to detect nucleic acids, coupled with the use of monoclonal antibodies (MAbs) to detect proteins, has led to significant changes in our understanding of the hepatitis B virus (HBV) carrier state and the pathogenesis of HBV-mediated chronic liver disease. Shortly after the National Institutes of Health (NIH) guidelines for recombinant DNA research were modified in 1979 to permit cloning of human pathogens, four laboratories reported on the cloning of HBV.[1–4] Methods were then developed rapidly to use molecular hybridization techniques to identify viral DNA sequences at the picogram or subpicogram level in the liver and serum of chimpanzee and human HBV carriers.[5–13] At the same time, high-affinity monoclonal antibodies to the hepatitis B surface antigen (HBsAg) were developed that were capable of detecting viral surface antigen in the 10 to 100 pg range.[14,15] These methods were then combined with molecular hybridization to show that some patients who were formerly thought to have cleared viral infection (because they had developed antibodies to all known viral proteins) or were infected with other viruses (because all conventional immunologic tests for HBV were negative), were in fact actively infected with HBV or "variant/mutant" forms of HBV.[16,17] Although there was some initial skepticism concerning these observations,[18] recent studies using the polymerase chain reaction (PCR) to amplify very small amounts of viral nucleic acids (approximately 10^{-17} to 10^{-18} pg) coupled with simplified DNA sequencing methods have confirmed and markedly extended our initial findings.[19–22]

In the early 1980s, it was observed that a large proportion of HBsAg carriers in a Greek population who had developed antibodies to the hepatitis B e antigen (HBeAg), as well as to hepatitis B core antigen (HBcAg), still produced and secreted substantial amounts of virus into the bloodstream.[23] In contrast to so-called benign carriers, who showed no liver pathologic changes other than the presence of "ground-glass" hepatocytes (hepatocytes in which the cytoplasm was full of HBsAg), a large proportion of these individuals also showed chronic liver disease.[24] Subsequent studies from Japan indicated that a specific subgroup of HBsAg carriers who were anti-HBe and HBV DNA positive in the serum had a highly virulent form of chronic active hepatitis with rapid progression to cirrhosis and death.[25] Why certain patients lost HBeAg and developed antibodies to this protein, which was initially thought to represent a serologic marker of viral replication, and yet these HBeAg-negative individuals still continued actively to replicate virus and developed chronic liver disease, remained a mystery. This article is directed at illustrating how modern molecular and immunologic methods have unraveled some of these mysteries and suggests the possibility that variant or mutant forms of HBV play a major role in the pathogenesis of liver disease associated with persistent viral infection.

INTRODUCTION

Studies over the last decade have shown that HBV may exist in serum and liver tissue at extremely low levels.[19–22] Viral latency and low level replication may occur even in the presence of antibodies to HBsAg (anti-HBs), HBcAg (anti-HBc), and HBeAg (anti-HBe).[20,21,26] These observations are the direct result of the introduction of highly sensitive and specific molecular hybridization and MAb techniques,[19,20,26,27] and it is now apparent that low level HBV and HBsAg has been found in some patients with chronic liver disease in the absence of any HBV serologic markers. Furthermore, the HBV genome shows a much higher mutation rate than previously recognized,[28] because the virus replicates through a reverse transcriptase mechanism similar but not identical to that of retroviruses.[29] Since reverse transcriptas s do not have

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proofreading capacity as part of their polymerase function, errors made during transcription of DNA from RNA are not corrected. The biologic consequences of mutations in the HBV genome identified to date include: (1) Viral latency within the hepatocyte;[28,30] (2) low-level replication;[19-22,26,28,30,31] (3) enhanced liver injury in some individuals with precore mutations;[32-34] (4) vaccine failure or nonresponsiveness due either to mutations in the surface antigen gene that allows the variant strain to escape prior HBsAg immunization or preexisting undetected low level infection;[35,36] (5) deletion mutants within the core and surface genes that may lead to or contribute to an abnormal immune response;[37,38] and (6) a previously unrecognized role of low-level infection as the cause of idiopathic liver disease.[19,22,26-28,30,31] These recent observations have been made possible by using new molecular methods to identify circulating encapsidated virions. In the most sensitive current version of these methods, virus particles are extracted from serum by MAb anti-HBs capture, followed by PCR amplification of HBV DNA or direct PCR amplification of viral nucleic acids extracted from serum and liver.[19,21,22] The molecular structure of some of these variants has been determined by cloning and DNA sequence analysis.[27,28,30-34,39] The functional and biologic characteristics of these variants are now being analyzed in many laboratories. For example, the biologic properties and replication competence of cloned full-length HBV genome derived from both serum and liver have been determined by transfection into replication competent hepatocellular carcinoma (HCC) cell lines followed by analysis of viral protein, RNA and DNA synthesis.[28,32-36,40] From a diagnostic point of view, it is now possible with these newer techniques to detect several viral particles per milliliter of serum.[19,21,22] Thus, it is now possible to assess the pathogenic role of low-level HBV and variant infection in patients with acute and chronic liver disease of uncertain etiology, even if there is no serologic evidence of past HBV infection.

Detection of Low-Level Hepatitis B Virus Infection in Serum

Figure 1 shows a rapid, sensitive, and specific method that is capable of detecting less than 10 HBV virion particles per 200 μl serum.[19] The method involves the capture of encapsidated viral particles from serum using a high affinity (4×10^{11} L/M) immunoglobulin M (IgM) monoclonal anti-HBs antibody coupled to a solid-phase support. The solid-phased support is then washed to remove serum proteins, which may act as inhibitors of the subsequent PCR. The solid-phase support is then heated to 95°C to release the viral DNA from the captured virions. PCR amplification is then initiated using specific primers of approximately 20 nucleotides derived from the core and precore sequences, which are highly conserved among the hepadnaviruses. This approach allows for amplification of all MAb anti-HBs captured virions and includes viral particles with substantial genomic heterogeneity. This technique is at least 1000-fold

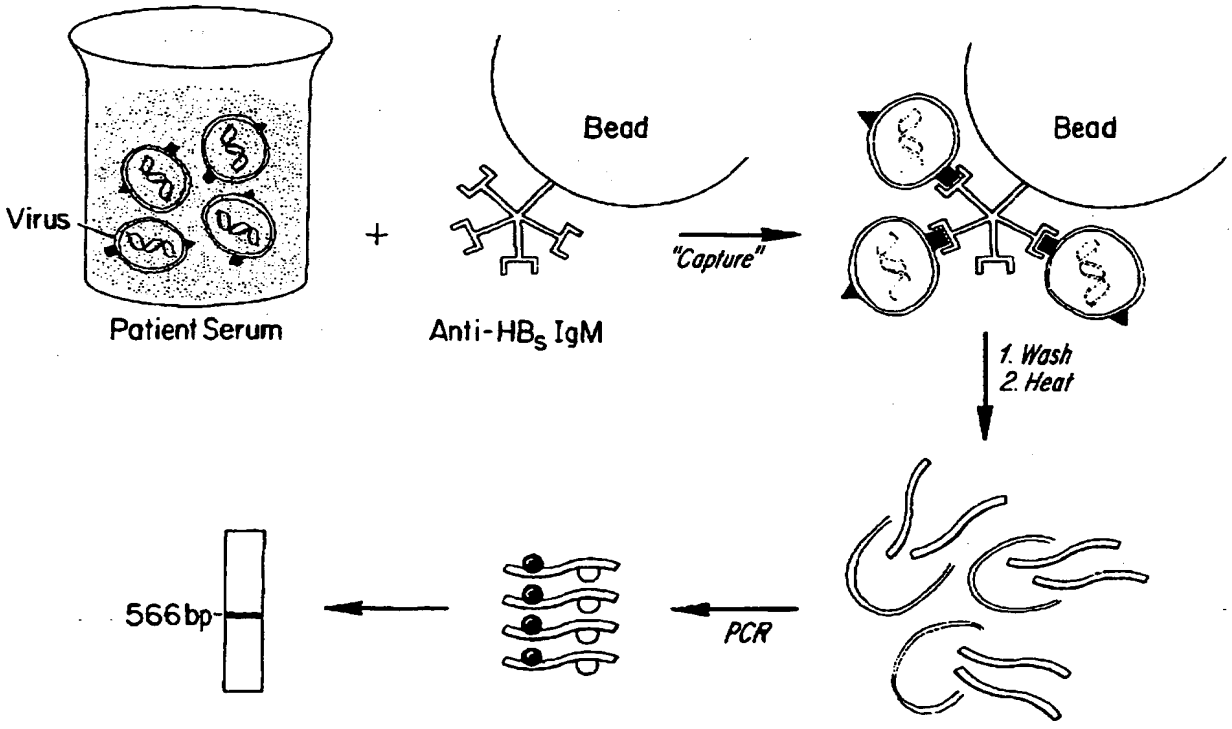

FIG. 1. Schema illustrating the detection of hepatitis B virus and/or variants in serum by monoclonal anti-HBs immunoglobulin M (IgM) capture followed by polymerase chain reaction (PCR) amplification.

FIG. 2. Analysis of 13 patients with chronic liver disease for the presence of hepatitis B virus (HBV)-related virions by anti-HBs immunoglobulin M capture/polymerase chain reaction amplification. Eleven of the 13 patients had no HBV serologic markers. However, patient 12 had anti-HBs and patient 11 had anti-HBc alone. All patients were negative for anti-HBsAg by conventional assays. Five individuals had persistent elevations of alanine aminotransferase (patients 1 through 5) and four others had cirrhosis of uncertain etiology (patients 6 through 9). The final four had chronic active (patients 10 and 11) and chronic persistent hepatitis (patients 12 and 13).

more sensitive than HBV DNA dot blot hybridization and monoclonal immunoradiometric assays (M-IRMA) for HBsAg-associated epitopes.[19] Using this method (Fig. 2), HBV DNA has been detected in both serum and liver of HBsAg-negative patients with and without anti-HBs and anti-HBc antibodies. These observations confirm and extend findings in the early 1980s in which approximately 35% of sera from patients with monoclonal anti-HBs reactivity, but negative tests for HBsAg by conventional polyclonal anti-HBs antibodies, were positive for HBV DNA by dot blot hybridization.[16] This illustrates the importance of a molecular approach to viral diagnosis.

Identification of Low-Level Hepatitis B Virus Infection in Hepatitis Vaccine Nonresponders

There are other circumstances in which detection of low-level HBV infection becomes important, as in the case of asymptomatic or unknown carriers in the general population. In order to investigate possible mechanisms for nonresponsiveness to HBV vaccination, low-level HBV infection has been identified by using the MAb capture/PCR amplification technique. It is noteworthy that, in immunocompetent subjects, approximately 5 to 10% of individuals fail to respond to vaccination. A population in Japan was studied to determine whether low-level HBV infection was responsible for some cases of HBV vaccine nonresponsiveness.[39] The hypothesis to be tested was that in a population with a known 3% HBV carrier rate, as determined by conventional serologic techniques, some cases of nonresponsiveness to hepatitis B immunization might be due in part to previously undetected low-level HBV infection. In this study, nine nonresponsive individuals had been identified from a vaccinated normal population; all nine had failed to develop an anti-HBs response, defined as an antibody level greater than a signal-to-noise ratio (S/N) of 2.1. These individuals had received three doses of 20 μg each of the HBV vaccine. A fourth booster was subsequently given to all nine "nonresponders." Table 1 summarizes the MAb PCR analysis and anti-HBs response to the booster vaccination. It is noteworthy that three individuals (O.Y., T.E., and O.A) responded to the booster dose and all were negative for low-level HBV by MAb/PCR analysis. In contrast, four of six individuals who did not respond to the booster dose were found to have low-level encapsidated HBV virions in serum, as shown by MAb/PCR analysis. Thus, these findings suggest that low-level infection with HBV may account for some nonresponsiveness to HBV vaccination. Therefore, one has to consider the possibility of low-level HBV infection in addition to other possible reasons, such as improper handling or administration of the vaccine, host genetic factors, and other diseases associated with immunosuppression, for lack of an inadequate anti-HBs response to HBsAg vaccination.

Detection of Low-Level Hepatitis B Virus Infection in Patients with Liver Dis ase f Unknown Etiology

Previous studies using DNA hybridization techniques and highly sensitive anti-HBs based M-IRMAs have identified HBV DNA and HBsAg in liver and serum of patients with chronic liver diseases with and without anti-HBs and anti-HBc who tested negative for HBsAg by conventional tests.[16,41-43] Furthermore, sera from such patients had been shown to transmit hepatitis infection to chimpanzees following intravenous inocu-

TABLE 1. Presence of Low-Level Hepatitis B Virus (HBV)-Related Viral Genomes in Serum and Anti-HBs Response to a Booster Dose of Anti-HBsAg in Vaccine Nonresponders

Patient	*Age*	*Sex*	*MAb/PCR**	*Anti-HBs Response*
T.C.	28	F	+	−
O.S.	35	M	+	−
K.F.	20	F	+	−
M.K.	24	F	+	−
I.N.	31	M	−	−
S.N.	22	F	−	+
O.Y.	35	F	−	+
T.E.	22	F	−	+
O.A.	47	F	−	+

*MAb/PCR: monoclonal antibody/polymerase chain reaction.

lation.[44,45] These experiments established that detection of HBV and variant infection by more sensitive techniques was biologically important. With the development of the PCR method to amplify viral DNA, it is now possible to clone and characterize viral molecules rapidly from low-level HBV and variant infections.

The clinical significance of low-level HBV is now being explored in well-defined groups of patients with chronic liver disease and cirrhosis. For example, sera from patients with HBsAg-negative chronic liver disease[26] have been analyzed for the presence of circulating encapsidated HBV using the MAb anti-HBs capture/PCR method (Table 2). All patients in this study had been tested for HBsAg by a sensitive M-IRMA (lower limit of detection was approximately 20 pg/ml HBsAg) and a less sensitive dot blot hybridization[46] for HBV DNA in serum and were found to be negative. As shown in Table 2, 31 and 29% of patients with liver disease in groups I and II, respectively (with and without anti-HBs), were reactive in the assay, whereas no individuals in the control groups (III and IV) were positive ($p < 0.0001$). Furthermore, independent assays performed on separate serum samples obtained 6 months later in 10 of 12 patients whose initial sera were positive remained positive, suggesting a chronic low-level carrier state.

Most of the 67 patients studied had undergone prior liver biopsy for clinical diagnosis and 42 paraffin blocks were available for extraction of DNA and subsequent PCR analysis. Table 2 also illustrates the results of these studies. It is of interest that 16 of 22 (73%) and 13 of 20 (65%) patients in groups I and II harbored HBV DNA sequences in their liver. Therefore all individuals with low levels of HBV virions in serum also had HBV DNA sequences in their liver. In contrast, there were some individuals with HBV DNA sequences in liver who had no detectable HBV virions in serum. This finding suggests that some patients with chronic liver disease may have latent viral genomes in hepatic tissue. The clinical consequences of this finding with respect to pathogenesis of liver injury awaits further study.

After identification of HBV-related viral genomes in serum and liver, direct analysis of the amplified viral sequences using restriction fragment analysis was performed. This additional method to explore the significance of HBV DNA in serum and liver was necessary for the following considerations: First, this approach provides additional evidence for the presence of HBV-related DNA sequences initially identified by the anti-HBs capture PCR amplification assay. Second, the amplified viral sequences may be directly analyzed with the known HBV DNA subtype sequences in order to compare their genomic organization. Third, this approach can exclude the possibility of contamination of the samples or assay reagents by a common viral DNA strain used in the laboratory. Figure 3 illustrates a rapid method for analyzing low-level HBV and variant genomes incorporating a restriction endonuclease step to generate a simple map of restriction fragments of the virus detected. The entire nucleic acid sequence of HBV subtypes is available and direct comparison between the subtypes revealed approximately 10% heterogeneity. By analyzing the patterns of restriction enzyme digestion of the amplified HBV viral genomes, it is possible experimentally to demonstrate genomic sequence homogeneity or heterogeneity. Since the pre-S and S regions demonstrate the most sequence variation among the subtypes, these regions were analyzed by restriction fragment polymorphism analysis, as shown in Figure 3. In this context, restriction fragment analysis of the PCR amplified liver and serum viral sequences from three patients was performed to compare the genomic organization of the virus derived from the liver and serum. As shown in Figure 4, the viral sequences were identical in serum and liver as shown by the patterns of restriction enzyme digestion. It is also apparent that the three viral genomes differ from each other, since they display subtle restriction enzyme fragment heterogeneity. This indicates infection by different HBV-related agents in each case and eliminates the possibility of cross-contamination of specimens.

Thus, a heterogeneous group of low-level HBV has been identified in patients with chronic liver disease previously thought to be unrelated to HBV infection. These studies illustrate the diagnostic importance of molecular techniques to establish the presence of low-level viral infection. It is also important to note that there are patients with idiopathic liver disease and amplifiable HBV DNA sequences in liver but no demonstrable HBV DNA in serum. Explanations for this phenomenon include the possibility that the level of circulating virions may fluctuate and fall below the detection limit of the anti-HBs capture/PCR assay or PCR amplification after viral DNA extraction from serum at any given time of sample acquisition. Second, in patients with anti-HBs, viral particles released from infected hepatocytes may be rapidly

TABLE 2. Prevalence of Low-Level Hepatitis B Virus (HBV) Infection in Patients with Chronic Liver Disease and Cirrhosis of Uncertain Etiology*

Group	Anti-HBs	Serum HBV DNA (Anti-HBs/PCR) No. Positive/No. Tested	%	Liver HBV DNA (PCR) No. Positive/No. Tested	%
I	−	11/36	31%†	16/22	73%
II	+	9/31	29%†	13/20	65%
III	−	0/32	0%	ND‡	
IV	+	0/23	0%	ND	

*The subjects with idiopathic liver disease (groups I and II) were analyzed for the presence of HBV DNA in both serum and liver and the controls with no liver disease (groups III and IV) in serum.
†$P < 0.0001$ comparing group I with group III and group II with group IV using Fisher's exact test.
‡ND = not done.

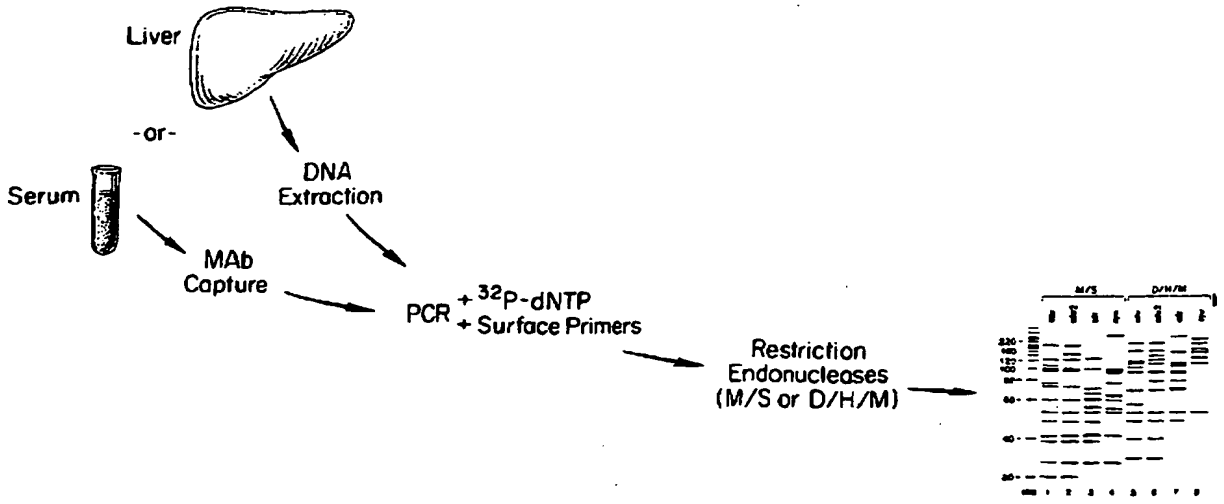

FIG. 3. DNA was extracted from liver using a standard method or from serum using the monoclonal antibody (MAb) capture/polymerase chain reaction (PCR) method and amplified in the presence of radioactive nucleotide precursors and primers spanning the surface region. The PCR product was subjected to digestion by a combination of restriction endonucleases (M/S or D/H/M). The digestion mixture was then electrophoresed and exposed to x-ray film. A computer-generated restriction analysis of various hepatitis B virus subtypes is shown.

removed from the circulation in the presence of high levels of anti-HBs. A third possibility is that the virus may be present in the liver in an episomal form without detectable transcription or replication. In this regard, the detection of latent HBV has been previously reported,[20,30,43,47,48] and the molecular mechanism for viral latency is now being explored (see later). Finally, the viral genome may be integrated into the hepatocyte DNA as a result of previous HBV infection, particularly in patients with anti-HBs antibodies. Additional studies will be necessary to distinguish between these various possibilities.

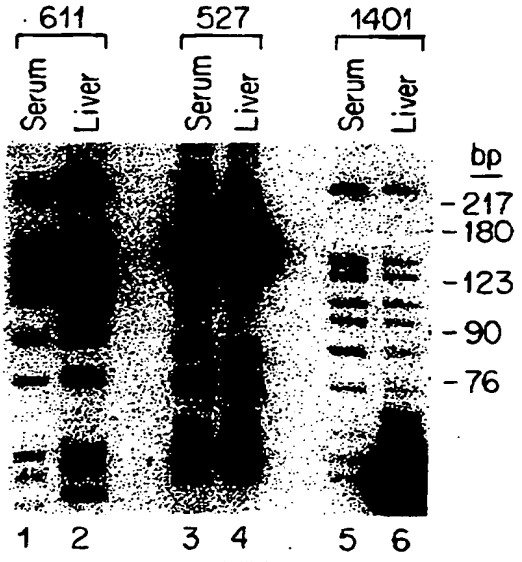

FIG. 4. Restriction-fragment analysis of hepatitis B virus DNA in serum and liver from three patients with cirrhosis. Note that the viral strain in the serum and liver from each patient is the same but there is subtle genomic variation of the virus isolated from each patient, indicating that they are distinct and separate viral strains.

Mechanism of Hepatitis B Viral Latency

The capability of amplifying low-level viral sequences by PCR in tissues such as liver has led to an exploration of the mechanisms of HBV latency following apparent serologic recovery from infection. One general approach has been to reconstruct full-length genomes from various PCR amplified subgenomic fragments of HBV DNA, followed by transfection of the viral DNA into a permissive human HCC cell line. These experiments allow the assessment of viral DNA, RNA, and protein synthesis by the reconstructed genome. If there are critical missense mutations within the viral genome that affect the viral replication cycle, this approach may functionally identify the important biologic role of the mutations. For example, HBV DNA species have been detected in serum and liver from HBsAg-negative individuals and experimental animals with and without antibodies to viral antigens, which illustrates the potential clinical importance of viral latency.[16,17,20,21,30,42–44,47,49,50] However, the fine structural details of these species have not previously been analyzed. Thus, to identify a possible genetic basis for the persistence of HBV DNA in HBsAg-negative patients, the HBV genome from the liver of a patient serologically immune to HBV infection[47] was characterized in detail. This patient had a history of viral hepatitis 23 years earlier. A full-length viral genome of 3221 bp was reconstructed from the pa-

tient's liver and the complete nucleotide sequence was established, as shown in Figure 5. At the structural level, numerous missense mutations were identified in all viral genes with the highest frequency in the precore/core gene (map position 1816–2462), which is otherwise the most conserved region among different HBV genomes and other hepadnaviruses.

Functional analysis of the mutant viral genome after in vitro transfection of a human hepatoma cell line revealed three major findings: (1) a low level of HBsAg synthesis, presumably caused by mutations affecting viral promoter-enhancer elements; (2) absence of precore/core protein in transfected cells and HBeAg in culture medium because of the in-phase stop codon mutation at the end of the precore region; and (3) incompetence to replicate because of a point mutation in the polymerase gene (arrow) that interfered with encapsidation of pregenomic RNA. Therefore the genetic basis of the replication defect was shown to be due to a single missense mutation in position 2798 in the 5′ region of the polymerase gene (Fig. 6, Table 3). Mutant viral DNA could be made replication competent by a C to A mutation in this position; conversely, "wild-type" HBV DNA was rendered replication defective by an A to C mutation in the same position. This mutation leads to substitution of proline for threonine in the N-terminal region of the polymerase, which is important for reverse transcription. By primer extension studies, it was further demonstrated that the molecular basis for the inability to replicate was a defect in the packaging of pregenomic RNA. In addition, complimentation studies demonstrated that the mutant viral genome could be complemented in *trans* by a full-length wild-type gene construct. In biologic terms, these findings suggest that the accumulation of mutant DNA in the patient's liver most likely occurred through *trans*-complementation by wild-

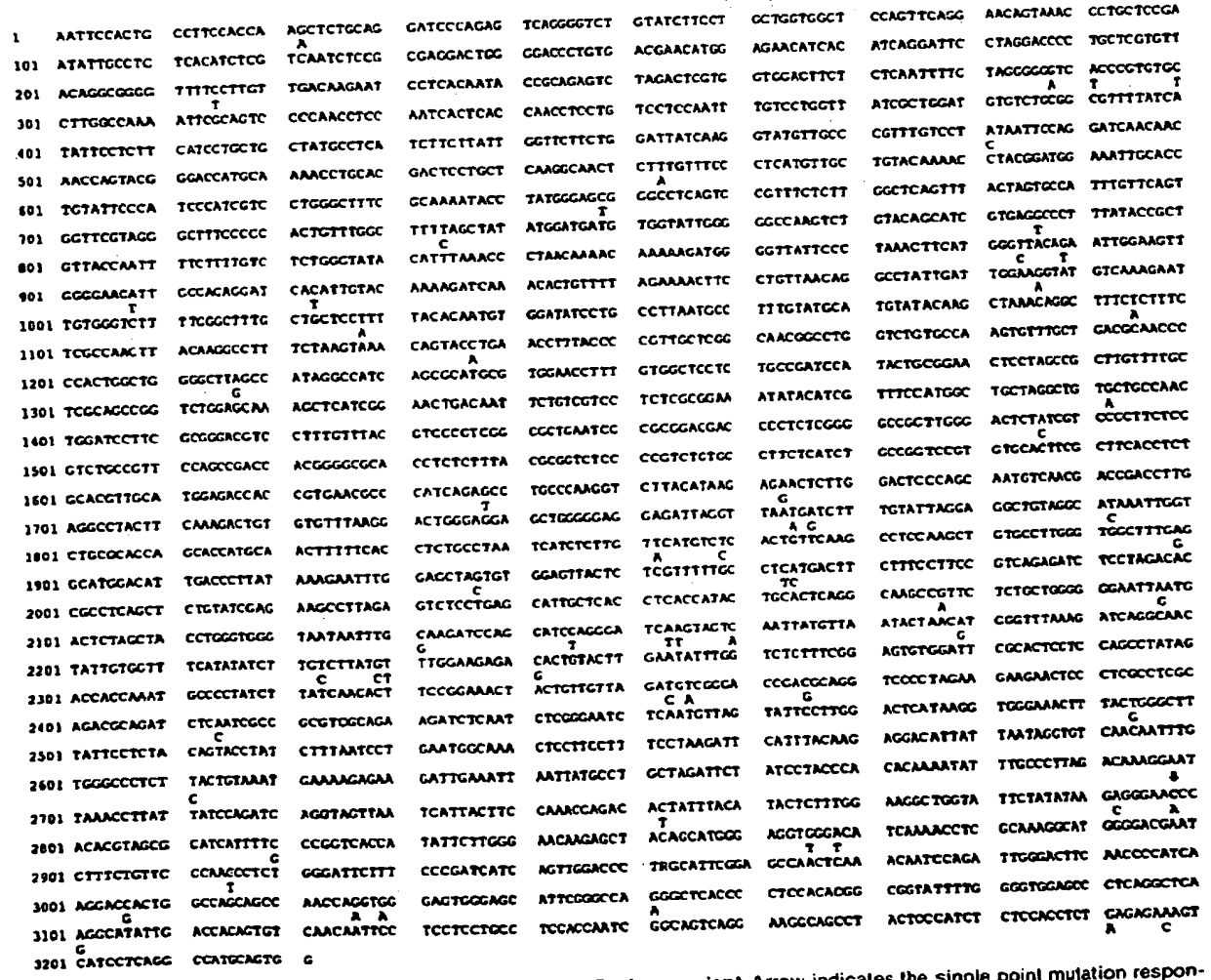

FIG. 5. Complete nucleic acid sequence of an hepatitis B virus variant. Arrow indicates the single point mutation responsible for the replication defectiveness of this virus. Numerous mutations in all viral genes are shown by the nucleotide sequences.

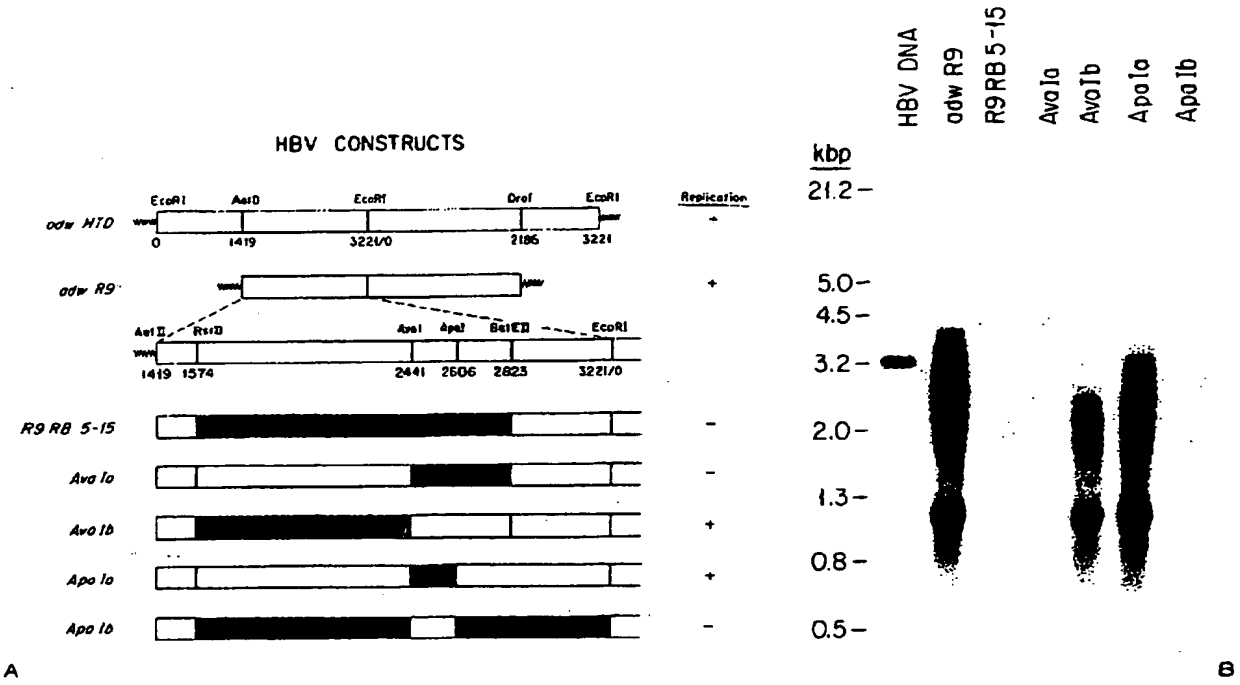

FIG. 6. A: Structure and replication competence of hepatitis B virus (HBV) subclones. The head-to-tail dimer of "wild-type" adw (adw HTD, cloned into the EcoRI site of pGEM-7) was subcloned as AatII (nt 1419) and DraI (nt 2186) fragments to yield the truncated, replication-competent construct adw R9. By exchange of specific regions between adw R9 and a mutant HBV genome (5-15), the subclones illustrated were obtained. The regions in black indicate HBV 5-15 insertions into adw R9. **B:** HBV DNA replication of HBV subclones. Southern blot analysis of cytoplasmic DNA isolated from HuH-7 cells 5 days after transfection with the clones indicated on top (see A). Autoradiographic exposure time was 1 day at −80°C; marker was 5 pg of HBV DNA.

type HBV DNA or HBV mutants with an intact polymerase gene. The mutation-induced changes in the biology of this variant virus, acquired during the 23-year period after initial infection with a presumably wild-type virus, may have allowed the HBV mutant to persist in liver cells by escaping from immune surveillance. Although wild-type and wild-type plus mutant-infected hepatocytes may have been eliminated by immune mechanisms directed against normal viral gene products, such as membrane-associated HBsAg[51] or HBc/eAg, the mutant R9R8 5-15 infected liver cells may have escaped immune elimination because of absent or low-level gene expression or mutation-induced antigenic changes of viral targets for the immune system. From this perspective, critical missense mutations may be a more general mechanism underlying HBV persistence through escape from immune surveillance or alteration of viral gene expression and replication.[52] Considering the persistence of viral DNA species in HBsAg-negative patients and experimental animals,[16,17,20,21,30,42-44,47,49,50] a detailed characterization of these viral DNA species, analogous to the strategy applied in this study, should allow assessment of the contribution of single missense mutations to the biology of HBV[21] and to the variable course of naturally

TABLE 3. DNA Sequence and Replication Competence of In Vitro Mutagenized Hepatitis B Virus (HBV) Clones and Their Parent Constructs

Clone	*DNA Sequence*				*Replication*
		2798	2820		
adw R9	2791-	GAGGGAAACC ACACGTAGCG	CATCATTTTG	CGGGT-2825	+
2-6	2791-	C		-2825	−
5-3	2791-		C	-2825	+
Ava Ia	2791-	GAGGGAAACCC ACACGTAGCG	CATCATTTTC	CGGGT-2825	−
6-6	2791-	A		-2825	+
3-3	2791-		G	-2825	−

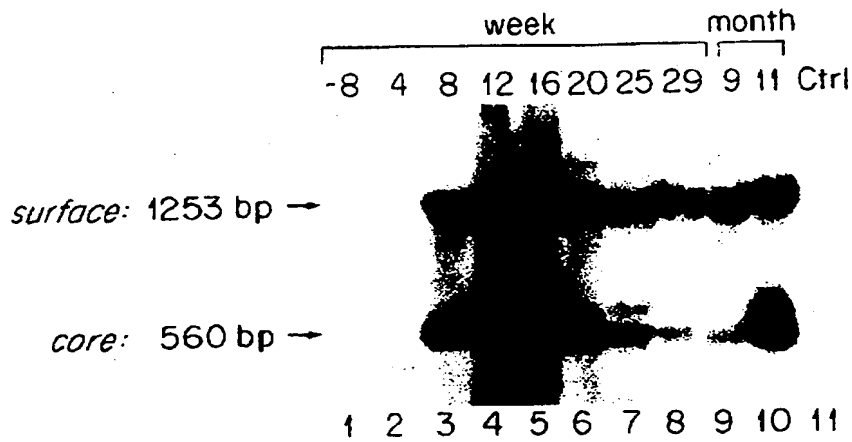

FIG. 7. Persistence of hepatitis B virus (HBV) DNA in chimpanzee liver following acute infection. Polymerase chain reactions using both core and surface primers were performed separately on extracted liver DNA and electrophoreses on the same lane and probed with ^{32}P-HBV DNA. Approximately 50 ng of total liver DNA was used in each reaction. Lane 1: 8 weeks before the time of inoculation (wk 0). The remainder are after inoculation as follows: lane 2, 4 weeks; lane 3, 8 weeks; lane 4, 12 weeks; lane 5, 16 weeks; lane 6, 20 weeks; lane 7, 25 weeks; lane 8, 29 weeks; lane 9, 9 months; lane 10, 11 months. Lane 11 is negative control.

occurring acute or fulminant and chronic HBV infection, including the development of HCC.

Evidence is accumulating that HBV variants, when studied in chimpanzee transmission experiments, may exhibit different biologic properties than wild-type viral strains.[53] For example, a recent study in a liver transplant recipient who was HBV positive at the time of transplantation revealed the evolution of an HBV variant during subsequent human anti-HBs IgM antibody administration to prevent reinfection of the donor liver. Under immunologic selection pressure from the exogenously administered IgM anti-HBs, a new HBV strain emerged with a point mutation leading to a glycine to arginine substitution in a highly conserved hydrophilic domain of HBsAg at amino acid position 145. This single amino acid change was sufficient to block immune reactivity with the IgM MAb and the patient became reinfected with the mutant strain. Inoculation of the variant virus into a chimpanzee demonstrated defective HBV replication and low-level HBsAg production in the liver. This study strongly supports a relationship between an induced amino acid difference in the HBsAg gene region, reduced MAb binding to the mutant strain, and defective viral replication in vivo.

In another HBV variant, serum from an individual with chronic liver disease and without HBV serologic markers but reactive by the MAb anti-HBs capture/PCR amplification was inoculated into a chimpanzee. After inoculation, an acute hepatitis B-like infection developed in the chimpanzee (Fig. 7). However, analysis of serial biopsy specimens, showed the persistence of HBV DNA for more than 17 months after resolution of acute hepatitis and seroconversion to anti-HBs positive. As shown in Figure 8, there was evidence that the viral DNA per-

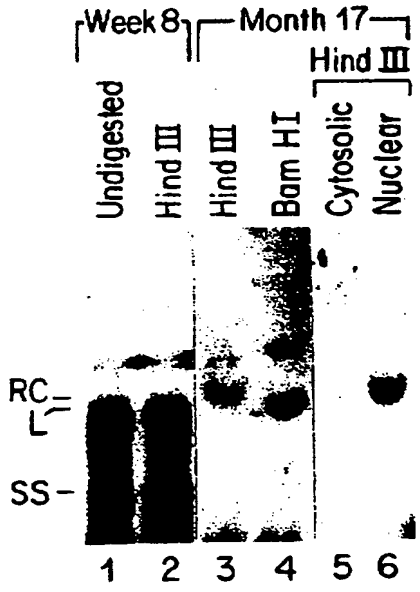

FIG. 8. Southern blot analysis of hepatitis B viru (HBV) DNA in infected chimpanzee liver. Liver DNA from week 8 (5 μg) and from month 17 (50 μg) were used for restriction enzyme analysis. DNA, 50 μg, from the nuclear fraction of hepatocytes (month 17) and equivalent amount from the cytosolic fraction were digested with Hind III. Lane 1, week 8, undig s-t d; lane 2, week 8, Hind III; lane 3, month 17, Hind III; lane 4, month 17, BamHI; lane 5, month 17, cytosolic fraction, Hind III; lane 6, month 17, nuclear fraction, Hind III. The positions for RC (relaxed circle), L (linear), and SS (single-stranded) forms of HBV DNA are shown.

TABLE 4. Significant Nucleotid and Amin Acid Substituti ns in an Hepatitis B Virus (HBV) Variant*

Pre-C/C Region			*Pre S-Region*			*X Region*			*S Region*		
Nucleotide†		*Amino Acid‡*	*Nucleotide†*		*Amino Acid‡*	*Nucleotide†*		*Amino Acid‡*	*Nucleotide†*		*Amino Acid‡*
1858/60	C→T	15 Pro→Ser	2929	A→G	27 Asp→Gly	1388	G→C	5 Val→Leu	164	A→G	3 Asn→Ser
2260	G→T	145 Leu→Phe	3010	A→C	54 Glu→Ala	1439	G→A	22 Gly→Ser	314	C→T	53 Ser→Leu
			3028	C→T	60 Ala→Val	1481	G→A	36 Ala→Thr	785	G→A	210 Ser→Asn
			3033	G→T	62 Ala→Ser	1514	G→A	47 Ala→Thr			
			3066/7	G/G→A/A	73 Gly→Asn	1632	A→G	86 His→Arg			
			8/9	C/A→G/C	125 Thr→Ser						

*The first nucleotide and amino acid are derived from the sequences of adr and the second are from those of the HBV variant. Mutations in the Pol region are not shown. There were a total of 34 amino acid substitutions.
†Nucleotide position is determined from position 0 at the EcoRI site in adr.
‡Amino acid position is numerated from the starting Met residue of each protein.

sisted in a nonreplicating episomal form (RC) in the nucleus of hepatocytes and the cytoplasm at 17 months was free of HBV replicative intermediates. This experimental chimpanzee infectivity study has demonstrated that, after clinical and serologic recovery from acute hepatitis infection, there may be persistence of low-level hepatitis B genomes in the liver despite the presence of anti-HBs. Recent investigations focusing on patients who have cleared HBsAg after apparently successful α-interferon treatment have found similar results.[54] In addition, cloning and sequencing analysis of residual genomes revealed multiple nucleotide and amino acid changes compared with all known hepatitis B virus subtypes (Table 4). These changes may have contributed in part to a different antigenic composition or led to persistence of the viral genome in liver tissue as a natural consequence of mutations occurring in important viral regulatory regions. It seems highly likely that the mutations detected in this variant viral genome were responsible for its aberrant biologic behavior, since this virus contained many significant mutations not found in other HBV strains. Further characterization of the biologic properties of this mutant strain, as well as additional HBV isolates, will be necessary to interpret the significance of mutations within various regions in the viral genome.

Evolution of Pre-S/S and Pre-C/C Deletion Mutants During Chronic Hepatitis B Virus Infection

Studies are now being performed in chronic carriers of HBV with special emphasis on mutations that occur in the pre-C/C region. In one study, chronic carriers of HBV without liver disease were analyzed for the presence of mutations within the pre-C/C region and comparisons were made to chronic carriers with chronic active hepatitis. It was found that approximately 70% of patients with chronic hepatitis had deletions within the core gene ranging from 105 to 183 nucleotides.[37] The presence of these deletions, as well as missense point mutations, correlated well with the development of liver injury. These deletion mutants were found to exist in more than half the patients who developed chronic liver disease. It will be of interest to determine whether these precore defective mutants alter lymphocyte cytotoxic responses, since it is unclear whether these pre-C/C deletion mutants are generated as a function of viral replication or are selected by the immune response following clearance of wild-type virions from the liver.

It has been suggested that HBV undergoes a higher rate of mutation than other DNA viruses during replication because of the infidelity of the reverse transcriptase. In a recent study, HBV DNA sequences in serum have been analyzed during a 4- to 6-year follow-up of an HBsAg-positive carrier.[36] It was found that there was an accumulation of missense mutations and deletions as well as rearrangements in the pre-S/S and pre-C/C genes during chronic HBV infection. Thus, mutant virions emerged in this patient during chronic infection; a finding that also has been observed by others.[45,53,55–57] The generation of defective viruses may be a function of ongoing viral replication; however, there may also be selection pressure toward the preservation of these mutated HBV strains due to altered host immune response. The mutated genomes may escape immune detection, depending on the specific location of point mutations, deletions, and rearrangements. Further study at the molecular level of HBV variant strains with viral deletions, mutations, and rearrangements will help clarify critical epitopes important for the immune response at both the T and B cell level and should help to identify the conditions necessary for viral clearance and generation of lymphocyte cytotoxic responses to virus in the liver.

Mutation in Genes Associated with Alter d Pathogenicity of Hepatitis B Virus

The HBeAg and HBcAg are encoded for the most part by the same sequence in the HBV genome in the same triplet reading frame (Fig. 9). HBeAg is synthesized initially as a precore molecule with 29 extra amino acids at the N-terminal region, using an AUG initiation codon 97 nucleotides upstream of the core AUG initiating codon. Nineteen of these extra amino acids are cleaved in processing of e antigen, as well as a large portion of the carboxy-terminal end, to yield a 16 kDa molecule in contrast to the 21 kDa mature core protein (Fig. 9). In various regions of the world, patients with severe and fulminant hepatitis have been found to harbor an HBV variant with a G to A mutation at nucleotide 1898 in the precore gene just upstream of the core AUG. This results in the generation of a stop codon in the pre-

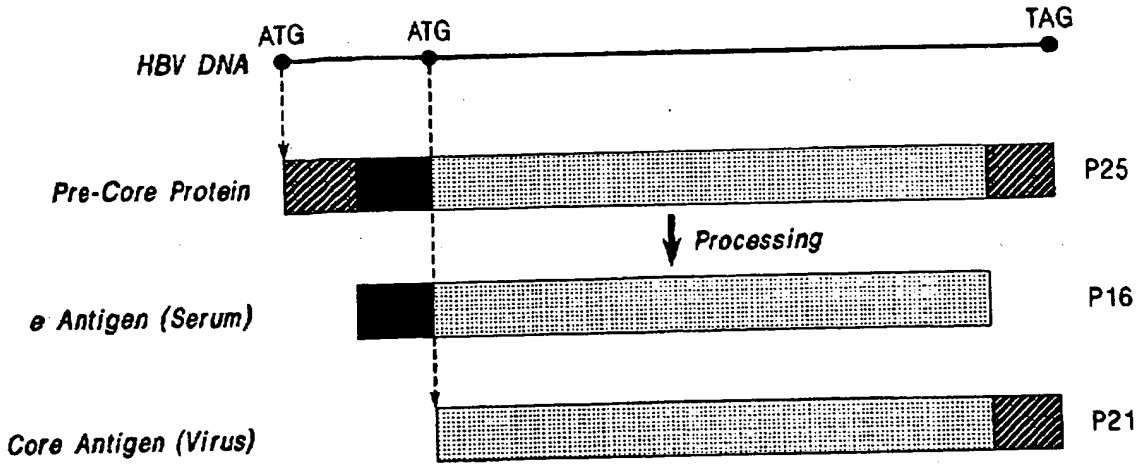

FIG. 9. Processing of e antigen from precore protein during hepatitis B virus (HBV) replication.

core open reading frame.[32-34,58-61] This mutation prevents the synthesis of the HBeAg but allows core antigen synthesis to continue normally and accounts for the serotype of HBsAg, HBV DNA and anti-HBe positive but HBeAg negative, originally identified in the early 1980s.[23,24] In other individuals, virions have been found to have an additional G to A mutation at nucleotide 1901 in the precore region and both of these mutations have been associated with fulminant hepatitis B.[32,34,58,61] These mutations have been found to be particularly common in Italy, Greece, Israel, and Japan. Indeed, both wild-type and mutant virus are often found in the same patient recovering from acute or chronic HBV infection. However, in fulminant hepatitis B cases studied in the United States and England, precore mutations have not been a consistent finding. The selection pressures that lead to emergence of a mutant strain are unknown. It seems clear, however, that some individuals who are exposed to HBV strains bearing these two precore mutations may be predisposed to a more severe form of liver injury, such as fulminant hepatitis.

An example of this phenomenon was illustrated by a nosocomial outbreak of five fatal cases of fulminant hepatitis B in Haifa, Israel.[34] Previous epidemiologic investigations had traced a suspected source to an anti-HBe-positive HBsAg carrier with chronic liver disease. The presence of HBV was identified by PCR amplification of viral DNA in serum derived from this individual, the five patients with fulminant hepatitis B, and from five individuals with acute self-limited hepatitis B as controls. The amplified viral DNAs were cloned and sequenced. These studies showed that an HBV variant with two mutations at positions 1898 and 1901 in the precore region was transmitted from the source patient to the five cases with fulminant hepatic failure. This HBV variant also contains significant sequence divergence in the X, precore, and core regions from all known HBV subtypes. Cloned HBV DNAs derived from a patient with a subclinical case of hepatitis B hospitalized at the same time as the outbreak and four other control subjects with acute self-limited hepatitis B all contained the wild-type sequence in the precore region (Fig. 10).

This study demonstrates transmission of a variant hepatitis B viral strain from a common source to five individuals who subsequently developed fulminant hepatitis B infection. Subsequent studies have shown that these two precore mutations do not appear to alter the synthesis of viral proteins, RNA or DNA. Moreover, creating these two mutations in the wild-type virus by in vitro mutagenesis also did not alter the biologic properties of the wild-type virus.[62] At present, the importance of these two unique precore mutations is at best a molecular marker for some HBV viral strains that may predispose to more severe liver injury. It is highly likely that other mutations within these viral strains may be more relevant to explain their altered biologic behavior in the host with respect to an association with severity of the viral hepatitis. The precise role of these HBV variants in producing severe liver injury, however, will require extensive molecular and clinical investigation.

Point mutations have been reported in the HBV surface antigen gene that may alter the pathogenicity and biologic properties of the mutant strain. In one recently described HBV variant, a point mutation from G to A at position 587 in the HBV genome resulted in an amino acid substitution from glycine to argenine.[36] It is within this region that the highly antigenic *a* determinant of HBsAg resides. This mutant strain infected 44 individuals, including infants of HBsAg carrier mothers, who had undergone both passive and active HBsAg immunization. Thus, this point mutation resulted in a vaccine escape mutant of HBV. Two other variant strains have also been reported to occur and replicate in the presence of vaccine-induced immunity. An HBsAg-negative serum derived from a patient with chronic liver disease has been found to transmit hepatitis to a chimpanzee previously vaccinated and subsequently shown to be immune by challenge with wild-type HBV.[44] This animal, al-

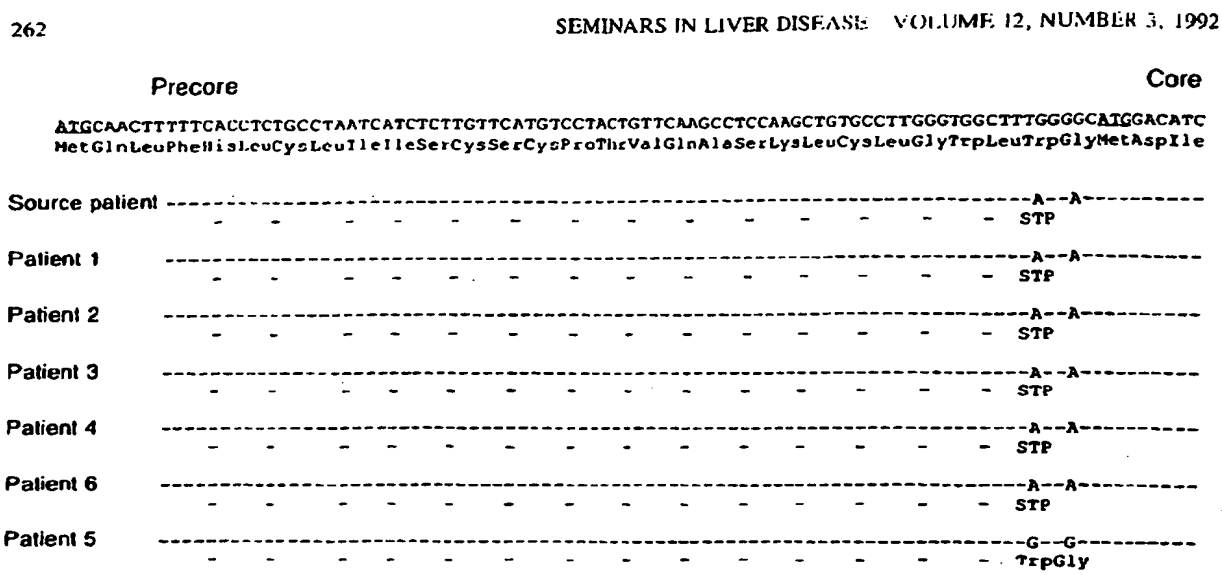

FIG. 10. Precore nucleotide and amino acid sequences of the fulminant hepatitis B strains. The nucleotide mutations and the resulting stop codon (STP) associated with the source patient and the five patients with fulminant hepatitis are shown. For comparison, the wild-type sequence of the asymptomatic patient (patient 5) is shown as well. Nucleotides 1816 to 1911 are shown.

though protected against the wild-type strain, was susceptible to infection by the mutant virus. Finally, in Senegal, a second mutant strain has been identified in individuals with vaccine-induced anti-HBs. They were thought to be infected with a novel strain designated HBV-2.[63] All three studies emphasize the importance of understanding mutations in the HBV surface antigen gene with respect to the evolution of vaccine escape mutants. It is unknown whether these mutant strains will become a more general problem and require different strategies for HBV vaccination in the future.

CONCLUSIONS

There seems to be little doubt that low-level HBV infection and variants play a significant role in the pathogenesis of acute and chronic inflammatory liver disease. If there is low-level HBV in serum, it is now possible to detect it directly by PCR amplification. If such agents are found in serum, a potential viral etiologic component in the pathogenesis of the liver injury should be considered. In addition, if liver biopsy material is available, extraction of nucleic acids followed by PCR can confirm the presence of the viral genome in the tissue even if fixed or paraffin embedded. Understanding the etiology of the liver injury may require the demonstration of the virus itself as the ultimate diagnostic test rather than the presence of anti-HBs, anti-HBc, and anti-HBe antibodies, which have been shown not to correlate with direct molecular markers of virus presence, especially in cases in which liver disease persists. Studies on the prevalence and molecular characterization of low-level HBV and variant infection are now possible by the techniques discussed in this review.

Mutation-induced changes in the viral genome represent a major focus of active investigation in laboratories throughout the world. In biologic terms, there is circumstantial evidence from molecular studies to suggest that HBV mutants may persist in liver cells by escaping from immune surveillance. From this perspective, critical missense mutations may be responsible for underlying HBV persistence through a variety of mechanisms, including escape from immune surveillance, alteration of viral gene expression and replication, or changes in the antigenic properties of HBsAg. Identification of patients with low-level HBV and variant infection may therefore be important not only in understanding the pathogenesis of their disease, including the cellular and humoral immune response to the virus, but may also lead to the development of new antiviral strategies to terminate viral replication in the liver.[64]

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